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(54) Title: CEA-BINDING PROTEINS AND METHODS FOR THEIR ISOLATION AND USE

(57) Abstract

Disclosed are proteins, or fragments thereof, that specifically bind carcinoembryonic antigen (CEA) in the presence of a divalent cation *in vitro*. Also disclosed are methods of isolating these and other proteins which bind CEA. In this method, a biological sample containing CEA and a CEA-binding protein (CBP) is provided and contacted with a divalent cation at a concentration and for a time sufficient to enable the binding of said CBP to CEA, thereby forming a CBP-CEA conjugate. The sample is then contacted with an adsorbent that binds CEA for a time sufficient to allow the conjugate to adsorb to the adsorbent. Any portions of the sample which have not adsorbed to the adsorbent are removed. The CBP is dissociated from the adsorbent-bound conjugate and collected.

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CEA-BINDING PROTEINS AND METHODS FOR THEIR  
ISOLATION AND USE

5 BACKGROUND OF THE INVENTION

The present invention relates to the field of cancer diagnosis and treatment, and more particularly to methods of detecting and treating carcinoma that elicit carcinoembryonic antigen (CEA). In particular, this invention relates to the identification of new cancer markers, CEA binding proteins, and methods of isolating such CEA-binding proteins, antibodies specific for these proteins, as well as methods useful in the diagnosis, detection, and monitoring of carcinoma.

Colorectal carcinoma is a cancer which affects approximately 600,000 additional people worldwide per year. The prognosis is poor in about 50% of the cases because the tumor is often not detected until the disease has spread and has reached a terminal stage. Early diagnosis is important to increase chances of arresting the carcinoma before it metastasizes, thereby leading to an improved prognosis.

A widely used method of the identification of cancerous tissue is to determine its structural resemblance to fetal or immature tissue. In this way, tumors can be classified depending on the degree 5 of cellular differentiation; they can be undifferentiated, poorly differentiated, moderately differentiated or well differentiated. In addition, the behavior of a given tumor can often be related to its degree of differentiation. For example, poorly 10 differentiated tumors tend to grow more rapidly and metastasize earlier than do differentiated tumors which more closely resemble the tissue of origin. Poorly differentiated tumors tend to have a poor prognosis and are difficult to detect.

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One method of early tumor diagnosis is detection of the presence of a marker or antigen specific for a particular tumor. These normally proteinaceous markers are synthesized by the tumor, 20 and may be found in the serum and/or on the surface of the tumor. Only a limited number of tumor markers for colorectal carcinoma have thus far been found to have clinical use. These include CEA and the sialylated Lewis a antigen (CA 19.9). Unfortunately, 25 approximately 40% of patients whose condition has been accurately diagnosed as colorectal carcinoma do not have elevated plasma levels of either of these antigens when initially examined. In the case of CEA, this may be because this antigen is so rapidly 30 cleared from the circulation. Recently, however, two new cancer markers, the carcinoma orosomucoid-related antigen (CORA) (U.S. Patent No. 4,921,789) and the and the CC-glycoprotein (U.S. Patent No. 4,914,021) have been discovered by applicants.

-3-

However, there is no commercially available serodiagnostic marker which can be used to detect the tumor and to monitor therapy for this group.

5 Production of some tumor markers e.g., CEA and CA 19.9, by tumor cells *in vitro* correlates with a greater degree of cellular differentiation. For example, CEA and CA 19.9 are present to a far lesser degree on poorly differentiated or undifferentiated 10 cancer cells than on those which are more differentiated. Accordingly, many patients with undifferentiated colorectal carcinomas never develop elevated serum levels of either of these markers, even in the terminal stages of the cancer. There is 15 also considerable overlap between the presence of CA 19.9 and CEA, the patient with a normal CEA level and an elevated level of CA 19.9 being the exception rather than the rule. Therefore, new markers suitable for identifying and monitoring 20 undifferentiated tumors would be of great value.

Accordingly, it is an object of this invention to provide new markers for the detection of carcinoma.

25 It is another object of the invention to provide new markers suitable for diagnosing and monitoring, and treatment of carcinoma.

30 Yet another object is to provide antibodies specific for these new markers.

Still another object is to provide a method of isolating CEA-binding proteins that eliminates contamination by CEA.

5           A further object of the present invention is to provide a method and kit for the detection and monitoring of carcinoma in patients using antibodies specific for markers on carcinoma cells.

10           Yet another object of the invention is to provide a hybridoma which produces a monoclonal antibody that recognizes both undifferentiated and poorly differentiated carcinoma cells.

15           A still further object is to provide screening procedures for detecting the presence of carcinoma cells at all stages of differentiation.

These and other objects of the invention  
20 will be apparent from the description, drawing and claims which follow.

SUMMARY OF THE INVENTION

New tumor markers for carcinoma have been discovered which have the ability to bind CEA. These 5 markers include two CEA-binding proteins (CBPs) having molecular weights of about 20,000 daltons (20 kD) and about 21 kD as determined by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), and binding CEA in vitro in the presence of a 10 divalent cation. The 20 kD protein includes the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:2. The 21 kD protein is glycosylated and includes the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:4.

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This invention also encompasses CEA-binding fragments of these tumor markers. The term "CEA-binding protein" or "CBP" is used herein to describe both proteins and fragments thereof which 20 bind CEA.

In one embodiment of the invention, the 20 kD or 21 kD CBP further includes a label, such as one selected from the group consisting of radioactive 25 isotopes, enzymes, stable free radicals, coenzymes, fluorescent groups, chemiluminescent groups, toxins, and colorimetric groups. In another embodiment, the 20 kD or 21 kD CBP is bound to a support which forms a device useful, for example, in purifying CEA.

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In yet another embodiment, antibodies and binding fragments thereof specific for a CBP are provided. Preferably, the antibody is a monoclonal antibody. The antibody can form part of a kit for

screening a patient for carcinoma. This kit further includes an antibody specific for a carcinoma marker selected from the group consisting of carcinoma orosomucoid-related antigen (CORA), the CC 5 glycoprotein, carcinoembryonic antigen (CEA), CA 19.9, non-specific cross-reacting antigen (NCA), and alpha 1-acid glycoprotein (AGP), serum amyloid P protein (SAP), and C-reactive protein (CRP).

10 The invention also provides methods of isolating and using CBPs. More specifically, a 15 method of isolating a CBP, such as the 20 kD, the 21 kD, or any CBP that binds CEA *in vitro* in the presence of a divalent cation, is provided which 20 includes the following steps. A biological sample containing CEA and a CBP is provided. The biological sample may be ascites fluid, whole blood, serum, bile, saliva, sputum, lymphoid tissue, or tumor tissue obtained from a subject afflicted with carcinoma. This sample is contacted with a divalent 25 cation, such as  $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$ , or  $\text{Mg}^{+2}$ , at a concentration and for a time sufficient to enable the CBP to bind to CEA, thereby forming a CBP-CEA conjugate. The sample is contacted with an adsorbent 30 that binds CEA, such as an immobilized immunoadsorbent including an antibody to CEA, for a time sufficient to allow the conjugate to adsorb to the adsorbent. Portions of the sample which have not adsorbed to the adsorbent are removed. The CBP is 35 then dissociated from the adsorbent-bound conjugate. Dissociation can be accomplished with the use of an agent that chelates divalent cations such as ethylenediaminetetraacetic acid (EDTA) or ethylene glycol-bis[ $\beta$ -aminoethyl ether]-N,N,N',N'-tetraacetic

acid (EGTA). The dissociated CBP is then collected, for example, by executing a purification method such as high pressure liquid chromatography (HPLC), SDS-PAGE, affinity chromatography, exclusion chromatography, ammonium sulfate precipitation, ultracentrifugation, and/or isoelectric focusing. For samples which do not contain CEA, CBP can be isolated by added CEA to the sample, or by reading the sample with CEA immobilized on a solid support in the presence of a divalent cation.

A method of detecting carcinoma is provided which includes the following steps. A pharmaceutical formulation including the 20 kD or 21 kD CBP, or 15 CEA-binding fragments thereof, is administered in a physiologically acceptable carrier to the subject. A biological sample is taken and the concentration of CEA assayed for CEA. The concentration of CEA is then compared with a predetermined threshold level of 20 CEA indicative of the presence of carcinoma.

Another aspect of the invention includes a method of screening for carcinoma including subjecting a biological sample from a subject to a 25 test, such as an immunoassay, indicating the presence of a cancer marker, and screening the sample for the presence of a CBP, its presence being indicative of the presence of carcinoma.

30 Other embodiments of the invention include assay formats detecting CEA in a body fluid by adsorbing CBP to a support (such as a microtiter plate, for example) treating the body fluid to be screen with divalent cation, adding the treated body

fluid to the CBP-bound plate, and then quantitating the bound CEA with labelled anti-CEA antibody. Alternatively, anti-CEA antibody can be adsorbed to the support which is then treated with the body 5 sample in the presence of divalent cation. The presence of complexed CEA (or CBP to which the CEA in the body sample is complexed in the presence of divalent cation) can then be detected by treatment with labelled anti-CBP antibody. CBPs free in body 10 samples can be detected by adsorbing CEA to a support, and then treating the support with the body sample in the presence of divalent cation.

Also provided is a method of treating 15 carcinoma. This method includes providing a CBP and administering it in a pharmaceutically acceptable carrier to a subject afflicted with carcinoma. The CBP binds CEA present in the subject, thereby inhibiting the metastatic proliferation of carcinoma 20 cells which occurs as a result of CEA binding to CEA receptors on various organs. The provision of the CBP can be accomplished by recombinant DNA technology, automated or manual biochemical peptide synthesis, or by purification from a subject 25 inflicted with carcinoma.

-9-

BRIEF DESCRIPTION OF THE DRAWING

The foregoing and other objects of this invention, the various features thereof, as well as 5 the invention itself may be more fully understood from the following description when read together with the accompanying drawings in which:

FIG. 1 is a diagrammatic representation of 10 the CBP purification scheme of the invention;

FIG. 2 is a photographic representation of a stained transfer blot of an SDS gel showing the 20 kD and 21 kD CBPs (lane 3), alpha 1-acid glycoprotein 15 (lanes 1 and 4), column wash (lane 2), and human serum albumin (lane 5);

FIG. 3 is an optical scan of an HPLC of (A) CEA, (B) CBP 41 kD complex, (C) CEA + CBP in the 20 presence of  $\text{CaCl}_2$ , and (D) CEA + CBP in the presence of EDTA;

FIG. 4 is an optical scan of an HPLC of (A) alpha 1-acid glycoprotein (AGP) + CEA in the presence 25 of EDTA, and (B) CEA + CBP in the presence of  $\text{CaCl}_2$ ;

FIGs. 5A and 5B are a comparison of the amino acid sequences of C-reactive protein and the 20 kD and 21 kD CBPs; and

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FIGs. 6A and 6B are a comparison of the amino acid sequences of serum amyloid P protein and the 20 kD and 21 kD CBPs.

DESCRIPTION OF THE INVENTION

Proteins that influence the concentration of CEA in the blood have been isolated from patients 5 afflicted with carcinoma. These CEA-binding proteins are also markers for carcinoma, and as such are useful in the detection and treatment of carcinoma.

The CBPs have been isolated using a 10 purification method which virtually eliminates contamination by CEA. This procedure is schematically represented in FIG. 1. A biological sample containing CEA and CBP is provided. The biological sample may be ascites fluid, whole blood, 15 serum, bile, saliva, sputum, lymphoid tissue, or tumor tissue obtained from a subject afflicted with carcinoma. This sample is contacted with a divalent cation, such as  $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$ , or  $\text{Mg}^{+2}$ , at a concentration and for a time sufficient to enable the 20 CBP to bind to CEA, thereby forming a CBP-CEA conjugate. The sample is contacted with an adsorbent that binds CEA, such as an immobilized immunoadsorbent including an antibody to CEA, for a time sufficient to allow the conjugate to adsorb to 25 the adsorbent. Portions of the sample which have not adsorbed to the adsorbent are removed. The CBP is then dissociated from the adsorbent-bound conjugate. Dissociation can be accomplished with the use of an agent that chelates divalent cations such as 30 ethylenediaminetetraacetic acid (EDTA) or ethylene glycol-bis[ $\beta$ -aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA). The dissociated CBP is then collected, for example, by executing a purification method such as high performance liquid chromatography (HPLC),

-11-

SDS-PAGE, affinity chromatography, exclusion chromatography, ammonium sulfate precipitation, ultracentrifugation, and/or isoelectric focusing.

5         The instant purification procedure does not depend on the size of the binding protein, a particularly important point since CBPs isolated by this method have a molecular weight (by HPLC) similar to that of alpha-1 acid glycoprotein (AGP). The 10 procedure is ligand-specific, and thus any proteins that may bind to CEA nonspecifically or which require some other binding factors will not contaminate the product. Any contaminants are removed during the 15 washing or will remain bound to CEA after the EDTA elution. This method also provides an effective way to isolate CBPs from CEA to which they have bound. Because CEA is a large molecule and very immunogenic, it is important to remove it from the CBP isolate if 20 antibodies to these CBPs are to be prepared from the isolate.

Using this method, two CBPs were isolated, one having a molecular weight of 20 kD and one 25 glycoprotein having a molecular weight of 21 kD, as shown in FIG. 2, lane 3, of a stained transfer blot of an SDS-PA gel. These molecular weights differ from those of other known CEA binding proteins such as the 46 - 50 kD carcinoma orosomucoid-related antigen (CORA) (described in U.S. Patent No. 30 4,914,021), and also differ from the 41 kD alpha 1-acid glycoprotein (AGP) which does not bind CEA.

The physical properties of the 20 kD and 21 kD CBPs, as well as the degree of purity after

isolation, were determined by high pressure liquid chromatography (HPLC). FIGS. 3A-3D show that the proteins isolated have a collective molecular weight of 41 kD (FIG. 3B). The appearance of a single peak with a retention time of about 8.5 min and the absence of a peak at about 9.4 min suggests the formation of a complex between CEA and CBP in the presence of calcium ions (FIG. 3C). However, in the presence of a calcium chelator like EDTA for example, two peaks with retention times similar to that of CEA (FIG. 3A) and that of the uncomplexed CBPs (FIG. 3B) were observed (FIG. 3D), indicating that these CBPs do not bind CEA in the absence of calcium.

These isolated CBPs were further distinguished from AGP by HPLC. Like the CBPs, AGP does not bind CEA in the absence of calcium (presence of EDTA) (FIG. 4A). However, unlike the CBPs, AGP does not bind CEA in the presence of calcium ions (FIG. 4B). These CBPs have also been distinguished from CRP and SAP by isoelectric focusing.

A partial sequence of the 20 kD and 21 kD CBPs was obtained and is set forth in the Sequence Listing as SEQ ID NO:2 and SEQ ID NO:4, respectively. These partial sequences were found to have no homology with CEA or AGP. However, some sequence homology was found with the serum amyloid P protein (SAP) (FIGS. 5A and 5B), and with the C-reactive protein (CRP) (FIGS. 6A and 6B). These sequence analyses were performed using the Wisconsin Program Protein Data Base (Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

-13-

SAP is a normal plasma component. It is an  $\alpha$ -1 glycoprotein composed of ten 23-25.5 kD subunits non-covalently linked as a double pentamer. The serum level of SAP in patients with various clinical 5 types of amyloidosis, connective tissue diseases, and bacterial pneumonia does not differ significantly from normal values. Likewise, the serum level of patients with carcinoma of the colon do not differ from healthy individuals in the serum level of SAP 10 (Levo et al. (1982) J. Immunol. Meth. 50:17-31). However, patients with carcinoma of the breast do have significantly increased serum concentrations of SAP which correlate with the severity of the disease (Levo et al. (Scand. J. Immunol. (1986) 24:147-151)).

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CRP, a dipentamer of 21 kD subunits, has a strong sequence homology with SAP. Both proteins undergo calcium-dependent ligand binding, are composed of non-covalently linked subunits, and share 20 a similar pentagonal disc-like molecular form (Pepys (1982) Eur. J. Rheumatol. Inflamm. 5:386). In addition, they share at least about 60% homology of amino acid sequence. However, unlike SAP, CRP is an acute phase reactant in humans. Its concentration 25 rises rapidly following acute tissue injury, infection, or inflammation, and it is often persistently elevated in cases of malignant neoplasia.

The sequence homology between the 20 kD and 30 21 kD CBPs, and CRP and SAP, respectively, raises the possibility that these proteins are related in structure and/or function as cancer markers.

Among the uses of the 20 kD and 21 kD CBPs are methods of detecting and treating carcinoma. The method of detection involves the administration to the subject of a pharmaceutical formulation including 5 the 20 kD or 21 kD CBP, or CEA-binding fragments thereof, in a physiologically acceptable carrier. A biological sample is then taken. This sample may be ascites fluid, whole blood, serum, bile, saliva, sputum, lymphoid tissue, or tumor tissue. The 10 concentration of CEA in this sample is measured. This measurement can be accomplished by any number of known tests for CEA including an immunoassay (e.g., Roche ELISA, Hoffman-La Roche, Nutley, NJ) activity assay, immunoassay, quantitative electrophoresis, and 15 the like. The concentration of CEA in the biological sample is then compared with a predetermined threshold level of CEA indicative of carcinoma. This threshold CEA concentration can be determined by administering a CBP to two statistically significant 20 groups of people, one with carcinoma, and one that is disease-free. By way of example, the value of the threshold level may be the point at which the measured CEA concentration curves of these two groups intersect.

25 CBPs may also be used in the treatment of carcinoma as follows. A CBP is administered in a pharmaceutically acceptable carrier to a subject afflicted with carcinoma. The CBP binds CEA present 30 in the subject, thereby inhibiting the metastatic proliferation of carcinoma cells which occurs as a result of CEA binding to CEA receptors on various organs (see, e.g., Toth et al. (1985) Cancer Res. 45:342-397; Toth et al. (1988) Biochem. Soc. Trans.

-15-

16:1027-1028; Toth et al. (1989) *J. Leukocyte Biol.* 45:370-376; and Hostetter et al. (1990) *J. Natl. Cancer Insti.* 82:380-385). The concentration of calcium in various body tissues is high enough to 5 enable efficient binding.

The provision of a CBP in both methods of use can be accomplished by purification from a subject inflicted with carcinoma. Alternatively, 10 given the sequence of these proteins isolated from body tissues, the CBPs may be prepared by recombinant DNA technology (see, e.g., Maniatis et al., Molecular Cloning, A Laboratory Manual (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), or by 15 automated or manual biochemical peptide synthesis.

Effective dosages of the CBPs and modes of their administration in the detection and treatment of carcinoma can be determined by routine 20 experimentation. The pharmaceutical formulation suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the 25 formulation must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms, such as 30 bacterial and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper

fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the 5 action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In some cases, it may be preferable to include isotonic agents, for 10 example, sugars or sodium chloride. Prolonged absorption of the injectable CBPs can be brought about by the use in the compositions of agents delaying absorption.

15            Sterile injectable solutions are prepared by incorporating the CBPs in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions 20 are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of 25 sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

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The CBP may be administered parenterally or intraperitoneally. Solutions of the CBP as pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as

hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations 5 contain a preservative to prevent the growth of microorganisms.

The CBP also may be orally administered, for example, with an inert diluent or with an assimilable 10 edible carrier, or enclosed in hard or soft shell gelatin capsules, or they may be compressed into tablets, or incorporated directly with the food of the diet. For oral administration, the CBP may be incorporated with excipients and used in the form of 15 ingestible tablets, buccal tablets, troches, capsules, elixirs, suspension syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of the CBP. The percentage of the compositions and preparations may, of course, be 20 varied. The amount of CBP in such useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and 25 the like may also contain the following: excipients, such as dicalcium phosphate; a disintegrating agent; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. 30 When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance,

tablets, pills, or capsules may be coated with shellac, sugar, or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent, methyl and propylparabens as preservatives, a 5 dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the CBP may be incorporated into 10 sustained-release preparations and formulations.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, 15 isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its 20 use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Antibodies to the 20 kD and 21 kD proteins 25 are also provided by the invention. Such antibodies can be easily produced by one with ordinary skill in the art. For example, a purified CBP isolate from a subject afflicted with carcinoma as an antigen can be used as an antigen. Mice, goats, rabbits, or other 30 animals can be challenged by injection with a solution of such an isolate emulsified in complete Freund's adjuvant at weekly intervals. After the initial injection, the booster injections can be administered without adjuvant or emulsified in

-19-

incomplete Freund's adjuvant. Alternatively, synthetic or genetically engineered analogs or fragments of the CBP produced by recombinant DNA or biochemical techniques can be used as immunogens. an 5 innoculum containing a relatively pure CBP sample isolated as described above along with Freund's adjuvant can be injected into a rabbit, mouse, rat, goat, or any mammal to produced monoclonal antibodies.

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Monoclonal antibodies to a CBP or active binding fragments of such antibodies can be generated by applying generally known cell fusion techniques (cf. Kohler and Milstein (1976) Eur. J. Immunol. 15 6:511-519; and M. Shulman et al. (1978) Nature 276:269-270, herein incorporated by reference) to obtain a hybridoma producing the monoclonal antibody. Optionally, the monoclonal antibody may be subjected to proteolysis to obtain an active Fab, 20 F(ab')<sub>2</sub>, or Fv fragment.

More specifically, monoclonal antibodies are prepared by obtaining mammalian lymphocytes (preferably spleen cells), committing the lymphocytes 25 to produce antibodies (e.g., by immunizing the mammal with the particular antigenic determinant of interest beforehand), fusing the lymphocytes with myeloma (or other immortal) cells to form hybrid cells, and then culturing a selected hybrid cell colony in vivo or in 30 vitro to yield antibodies which are identical in structure and specificity.

In particular, monoclonal antibodies to a CBP can be raised by employing a purified CBP isolate

-20-

from a subject afflicted with carcinoma as an antigen. Mice or other animals can be challenged by injection with a solution of such whole cells emulsified in complete Freund's adjuvant at weekly 5 intervals. After the initial injection, the booster injections can be administered without adjuvant or emulsified in incomplete Freund's adjuvant. Alternatively, synthetic or genetically engineered 10 analogs or fragments of the CBP produced by recombinant DNA or biochemical techniques can be used as immunogens.

Serum samples from the immunized animal can be analyzed by an enzyme linked immunoabsorbent assay 15 ("ELISA") or the like for antibody reaction with the immunization agent. Animals that exhibit antibodies titers are sacrificed and their spleens homogenized. Alternatively, the spleen cells can be extracted and the antibody-secreting cells expanded in vitro by 20 culturing with a nutrient medium. The spleen cells are then fused with myeloma (or other immortal) cells by the above-referenced procedure of Kohler and Milstein. The hybridomas so produced are screened (i.e., cloned by the limiting dilution procedure of 25 the above-referenced Baker et al. article) to select a cell line producing antibodies which react with human  $\alpha$  chain receptor proteins.

Large scale antibody production can be 30 obtained from such anti-CBP-producing cell lines by various techniques, including the induction of ascites tumors (e.g., after priming with pristane) and the purification of such antibodies from the ascites fluid by Protein A-Sepharose affinity

chromatography. For a further description of general hybridoma production methods, see Oi and Herzenberg, "Immunoglobulin-Producing Hybrid Cell Lines" in Selected Methods in Cellular Immunology (Mishell and Shiigi, Ed., W.H. Freeman & Co., 1980); and Scearce and Eisenbarth (1983) Meth. Enzymol. 103:459-469; and U.S. Patent 4,411,933 issued to Gillis on October 25, 1986, herein incorporated by reference.

10 Human antibodies (i.e., those obtained from human-human or human-animal hybridoma) can be used as well as animal antibodies. For descriptions of human hybridoma production techniques, see U.S. Patent No. 4,451,570 issued to Royston et al. on May 29, 1984; 15 U.S. Patent No. 4,529,694 issued to Lazarus et al. on July 16, 1985 and Zurawski et al., "Continuously Proliferating Human Cell Lines Synthesizing Antibody of Predetermining Specificity" in Monoclonal Antibodies (Plenum Press, New York 1980), also 20 incorporated by reference.

Active antibody fragments can be derived from the monoclonal antibodies disclosed herein by a number of techniques. For example, purified 25 monoclonal antibodies can be cleaved with an enzyme such as pepsin, and then subjected to HPLC gel filtration. The appropriate fraction containing Fab, F(ab)<sub>2</sub>, or Fv can then be collected and concentrated by membrane filtration or the like. For further 30 description of general techniques for the isolation of active fragments, see for example, Khaw, et al., Vol. 23 J. Nucl. Med., pp. 1011-1019 (1982), incorporated by reference.

The antibodies and antibody fragments used herein can be labeled preferably with radioactive labels by a variety of techniques other than the above-described Baker et al. technique. For example, 5 the biologically active molecules can also be labeled with a radionucleotide via conjugation with the cyclic anhydride of diethylenetriamine penta-acetic acid (DTPA) or bromoacetyl aminobenzyl ethylamine diamine tetra-acidic acid (BAME). See Hnatowich et 10 al., Vol. 220 Science, pp. 613-615 (1983) and Meares et al. (1984) Analytical Biochemistry, Vol. 142, pp. 68-78, incorporated by reference for further description of labeling techniques.

15 The instant invention also relates to a method for screening subjects for carcinoma. It includes subjecting a biological sample to at least one test selected from a plurality of tests, each of which is specific for a carcinoma cell. Useful 20 biological samples include ascites fluid, whole blood, serum, bile, lymphoid tissue, or tumor tissue obtained from a subject afflicted with carcinoma. The method used to obtain these samples is dependent on the nature of the sample and would be known by a 25 medical practitioner. Each test correlates the presence of a specific marker with the presence of a carcinoma cell, and in some instances, with a degree of differentiation of that cell.

30 The screening method of the present invention includes tests for tumor markers CEA, CA 19.9, NCA, AGP, CORA ( U.S. Patent Application Ser. No. 441,368), and the CC glycoprotein ( U.S. Patent No. 4,921,789), as well as any additional markers

-23-

which indicate the presence of carcinoma, such as CRP or SAP. The tests may be performed in a sequential manner until the presence of at least one marker has been proven.

5

The tests performed may be assays, for example, to determine enzyme-linked activity, or may be immunoassays which utilize an antibody specific for a particular marker (see, e.g., U.S. Patent No. 10 4,892,933). For example, an antibody raised to a cancer marker can be adhered to an adsorbent via chemical modification and/or covalent linkage using a bifunctional cross-linking reagent.

15

This invention provides a convenient kit for screening biological samples for colorectal carcinoma. This kit includes antisera or purified polyclonal or monoclonal antibodies specific for the 20 kD and 21 kD CBPs, and antibodies for at least one other tumor marker such as the CC glycoprotein, CORA, NCA, CA 19.9, CEA, AGP, SAP, or CRP. These antibodies can be prepared by methods well known to those skilled in the art (see description above). Of course this kit may contain antigen binding fragments 25 of such antibodies such as Fv, Fab, or F(ab)<sub>2</sub> fragments obtained by known proteolytic cleavage or recombinant DNA techniques. Screening may be performed by any immunoassay procedures known in the art such as, for example, radioimmunoassay, Western blot analysis, or nitrocellulose "dot" analysis.

The following examples illustrate the best mode of making and practicing the present invention,

-24-

but are not meant to limit the scope of the invention, since alternative methods may be used to obtain similar results.

5

EXAMPLE 1  
Purification of CBPs

CBPs were isolated from ascites fluid from patients with colorectal adenocarcinoma using the following procedure. Exogenous  $\text{CaCl}_2$  was added to human ascites fluid to give a final concentration of 5 mM. The ascites was then incubated with an anti-CEA monoclonal antibody (Hybritech, San Diego, CA) coupled to an affinity gel (Affi-Gel 10; Bio-Rad). After an overnight incubation with agitation at 4°C, the immunoadsorbent plus ascites fluid was put onto a (Affigel 10, Biorad, Richmond, CA) column, and any unbound material allowed to flow into a waste tube. The column was washed with wash buffer containing calcium (0.1 M Tris, pH 7.4, 0.15 M NaCl, and 5 mM  $\text{CaCl}_2$ ) to remove any unadsorbed molecules. CBP was eluted from the column using elution buffer containing EDTA (0.1 M Tris, pH 7.4, 0.15 M NaCl, 10mM EDTA).

25

EXAMPLE 2  
Analytical Methods

A. HPLC

30

CBP extracted from human ascites fluid as described above was passed through a size exclusion column (GF-250, DuPont). The eluates were analyzed

-25-

with the mobile phase (0.2 M sodium phosphate, pH 7.0, 0.005% sodium azide) at a flow rate of 1 ml/min and detection at 280 nm. The resulting elution profiles are shown in FIGS. 3A-3D. CBP migrates 5 with CEA in the presence of calcium (FIG. 3C), while in the absence of calcium (presence of EDTA), it migrates as a separate peak (FIG. 3D).

#### B. Protein Sequencing

10 Samples were prepared for amino acid sequence analysis as follows. CBPs purified as described in EXAMPLE 1 were electrophoresed on an SDS-polyacrylamide gel (10-20% Mini-Gel, ISS, Newton, 15 MA) according to the method of Laemmli (Nature (1970) 227:680-685). The proteins on the gel were then electrotransferred to an Immobilon <sup>TM</sup>P Transfer Membrane (Millipore) with transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 15% methanol v/v) 20 according to the method of Towbin et al. (Proc. Natl. Acad. Sci. (USA) 76:4350-4354). After staining the membrane with Coomassie Blue, it was washed several times with distilled water to remove traces of transfer buffer. The photograph of the stained 25 membrane is shown in FIG. 2. Two proteins are present having molecular weights of 21 kD and 20 kD.

A duplicate gel was stained with periodic acid-Schiff base reagent (PAS) according to the 30 method of Barber et al. (Biochem, (1971) 10:4711). This procedure demonstrated that the 21 kD protein is glycosylated.

-26-

The CBP bands were excised from the gel and mechanically sequenced according to the method of Matsudaria (J. Biol. Chem. (1988) 262:10035-10038) using an Applied Biosystems 477A protein sequencer. 5 The resulting sequence obtained from the 20 kD CBP is set forth in the Sequence Listing as SEQ ID NO: 2. The sequence obtained from the 21 kD CBP is shown in the Sequence Listing as SEQ ID NO: 4.

10

EXAMPLE 3

## Binding Assays

Affinity purified CBP was tested for the ability to bind to CEA in the presence of calcium 15 ions as follows. Varied amounts of CBP was mixed with 5-10  $\mu$ l CEA (100  $\mu$ g/ml) CEA in the presence of 5 mM  $\text{CaCl}_2$ . The sample was then run on an HPLC sizing column (DuPont GF-250), and the elution profile was recorded (FIG. 3C).

20

The appearance of a single peak with a retention time of about 8.5 min and the absence of a peak at about 9.4 min suggests the formation of a complex between CEA and CBP. In control experiments 25 (FIG. 3D) where CEA and CBP were mixed in the absence of  $\text{Ca}^{2+}$  (EDTA added), two peaks with retention times similar to that of CEA (FIG. 3A) and that of CBP (FIG. 3B) were observed.

30

EXAMPLE 4  
Isoelectric Focusing

The 20 kD CBP, the 21 kD CBP, CRP, and SAP were radioiodinated with  $^{125}\text{I}$  using the chloramine T

-27-

procedure of Greenwood et al (Biochem. J. (1963) 89:114-123) to procure a specific activity of about 6-10 mCi/mg. Focusing was carried out in agarose gels essentially as described by Saravis et al 5 (Immunol. Meth. (1979) 29:91-96). Radiolabelled protein was detected by audoradiography using Kodak X-OMAT film.

EXAMPLE 5

10

Assay for CEA

NUNC-Immuno Plates (Naperville, IL) were coated with 500 ng/well CEA mAb in carbonate buffer (0.05 of sodium carbonate, ph 9.6), and were 15 incubated overnight (ON) at 4°C. The plates were washed with phosphate buffered saline (PBS) containing 1% (wt/vol) bovine serum albumin (BSA) and 0.5% (vol/vol) Tween 20 (PBS-BSA-TWEEN). They were then treated with 2% BSA in PBS for two hours at 4°C 20 to block any remaining sites on the plate. Antigen and high CEA serum standards were diluted and added to the appropriate wells at 50 µl/well. The plates were incubated ON at 4°C, and then washed with PBS-BSA-TWEEN buffer. 50 µl/well biotin-labelled 25 anti-CEA monoclonal antibody (lot no. E50713-103; 2.5 µg/ml) was added and incubated for 3 hours at 37°C. The plates are washed with PBS-BSA-TWEEN. Stepavidin peroxidase-conjugated horse radish peroxidase (HRP) was added (diluted according to the concentration of 30 the lot), and the plate incubated for 1 hour at 37°C. The plates were then washed with PBS-BSA-TWEEN. They were developed with orthophenylenediamine (Sigma, St. Louis, MO) and read with a spectrophotometer at 495 nm.

-28-

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all 5 respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are 10 therefore intended to be embraced therein.

-29-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Maswoswe, Sibusisiwe M.

5 Briggman, Joseph V.

Toth, Carol A.

Thomas, Peter

(ii) TITLE OF INVENTION: Method for Isolating  
CEA-Binding Protein

10 (iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Lahive &amp; Cockfield

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(C) CITY: Boston

15 (D) STATE: Massachusetts

(E) COUNTRY: U.S.A.

(F) ZIP: 02109

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 720kb storage

20 (B) COMPUTER: IBM XT

(C) OPERATING SYSTEM: DOS 3.30

(D) SOFTWARE: Word Perfect 5.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 708,885

25 (B) FILING DATE: 31 May 1991

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

30

-30-

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 225 amino acids

(B) TYPE: amino acid

5 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (vii) FEATURE:

(A) NAME: Serum amyloid P component

## (x) PUBLICATION INFORMATION:

10 (A) AUTHORS: Mantzouranis, Evangelia C.

Downton, S. Bruce

Whitehead, Alexander S.

Edge, Michael D.

Bruns, Gail A. P.

15 Colten, Harvey R.

(B) TITLE: Human Serum Amyloid P Component

(C) JOURNAL: J. of Biological Chemistry

(D) VOLUME: 260

(E) ISSUE: 12

20 (F) PAGES: 7752-56

(G) DATE: 25 JUN 1985

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

25 Met Glu Lys Leu Leu Leu Cys Phe Leu Val Leu  
5 10Thr Ser Leu Ser His Ala Phe Gly Gln Thr Asp  
15 2030 Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu  
25 30Ser Asp Thr Ser Tyr Val Ser Leu Lys Ala Pro  
35 35 40

-31-

Leu Thr Lys Pro Leu Lys Ala Phe Thr Val Cys  
45 50 55

Leu His Phe Tyr Thr Glu Leu Ser Ser Thr Arg  
5 60 65

Gly Tyr Ser Ile Phe Ser Tyr Ala Thr Lys Arg  
70 75

10 Gln Asp Asn Glu Ile Leu Ile Phe Trp Ser Lys  
80 85

Asp Ile Gly Tyr Ser Phe Thr Val Gly Gly Ser  
90 95

15 Glu Ile Leu Phe Glu Val Pro Glu Val Thr Val  
100 105 110

Ala Pro Val His Ile Cys Thr Ser Trp Glu Ser  
20 115 120

Ala Ser Gly Ile Val Glu Phe Trp Val Asp Gly  
125 130

25 Lys Pro Arg Val Arg Lys Ser Leu Lys Lys Gly  
135 140

Tyr Thr Val Gly Ala Glu Ala Ser Ile Ile Leu  
145 150

30 Gly Gln Glu Gln Asp Ser Phe Gly Gly Asn Phe  
155 160 165

Glu Gly Ser Gln Ser Leu Val Gly Asp Ile Gly  
35 170 175

Asn Val Asn Met Trp Asp Phe Val Leu Ser Pro  
180 185

40 Asp Glu Ile Asn Thr Ile Tyr Leu Gly Gly Pro  
190 195

Phe Ser Pro Asn Val Leu Asn Trp Arg Ala Leu  
200 205

45 Lys Tyr Glu Val Gln Gly Glu Val Phe Thr Lys  
210 215 220

50 Pro Gln Leu Trp Pro  
225

-32-

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 amino acids

(B) TYPE: amino acid

5 (D) TOPOLOGY: linear

(iii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

10 Thr Asp Leu Ser Gly Lys Val Phe Val Phe Pro  
5 10Glu Ser Val Thr Asp Val Asn Leu Ile Thr Pro  
15 2015 Leu Glu Lys Pro Leu Gln Phe Thr Ser Ala Tyr  
25 30

## 20 (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 223 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

## (vii) FEATURE:

(A) NAME: C-reactive protein

## (x) PUBLICATION INFORMATION:

(A) AUTHORS: Lei, Ke-Jian

30 Liu, Teresa

Zon, Gerald

Soravia, Emilia

Liu, Teh-Yung

Goldman, Neil D.

35 (B) TITLE: Genomic Sequence for Human  
C-reactive Protein

-33-

(C) JOURNAL: J. of Biological Chemistry

(D) VOLUME: 260

(E) ISSUE: 24

(F) PAGES: 13377-83

5 (G) DATE: 25 OCT 1985

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Asn Lys Pro Leu Leu Trp Ile Ser Val Leu  
10 5 10

Thr Ser Leu Leu Glu Ala Phe Ala His Thr Asp  
15 15 20

15 Leu Ser Gly Lys Val Phe Val Phe Pro Arg Glu  
25 25 30

Ser Val Thr Asp His Val Asn Leu Ile Thr Pro  
35 35 40

20 Leu Glu Lys Pro Leu Gln Asn Phe Thr Leu Cys  
45 50 55

Phe Arg Ala Tyr Ser Asp Leu Ser Arg Ala Tyr  
25 60 65

Ser Leu Phe Ser Tyr Asn Thr Gln Gly Arg Asp  
70 75

30 Asn Glu Leu Leu Val Tyr Lys Glu Arg Val Gly  
80 85

Glu Tyr Ser Leu Tyr Ile Gly Arg His Lys Val  
90 95

35 Thr Ser Lys Val Ile Glu Lys Phe Pro Ala Pro  
100 105 110

40 Val His Ile Cys Val Ser Trp Glu Ser Ser Ser  
115 120

Gly Ile Ala Glu Phe Trp Ile Asn Gly Thr Pro  
125 130

-34-

Leu Val Lys Lys Gly Leu Arg Gln Gly Tyr Phe  
135 140

5 Val Glu Ala Gln Pro Lys Ile Val Leu Gln Gly  
145 150

Glu Gln Asp Ser Tyr Gly Gly Lys Phe Asp Arg  
155 160 165

10 Ser Gln Ser Phe Val Gly Glu Ile Gly Asp Leu  
170 175

Tyr Met Trp Asp Ser Val Leu Pro Pro Glu Asn  
180 185

15 Ile Leu Ser Ala Tyr Gln Gly Thr Pro Leu Pro  
190 195

Ala Asn Ile Leu Asp Trp Gln Ala Leu Asn Tyr  
200 205

20 Glu Ile Arg Gly Tyr Val Ile Ile Lys Pro Leu  
210 215 220

25 Val Trp Val

## (2) INFORMATION FOR SEQ ID NO:4:

30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

35 (vii) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Gly Gln Thr Leu Met Gly Gly Lys Ala Phe  
5 10

40 Val Phe Pro Lys Ser  
15

-35-

We claim:

1. A method of isolating a protein which binds  
5 carcinoembryonic antigen comprising the steps of:

(a) providing a biological sample  
containing carcinoembryonic antigen (CEA) and  
a CEA-binding protein (CBP);

10 (b) contacting said sample with a divalent  
cation, such as  $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$ , or  $\text{Mg}^{+2}$ , at a  
concentration and for a time sufficient to  
enable the binding of said CBP to CEA,  
15 thereby forming a CBP-CEA conjugate;

(c) contacting said sample with an adsorbent  
that binds CEA for a time sufficient to allow  
said conjugate to adsorb to said adsorbent;

20 (d) removing portions of said sample which  
have not adsorbed to said adsorbent;

25 (e) dissociating said CBP from said  
adsorbent-bound conjugate; and

(f) collecting said dissociated CBP.

2. The method of claim 1 wherein said providing  
30 step comprises obtaining a body sample, such as  
ascites fluid, whole blood, serum, bile, saliva,  
sputum, lymphoid tissue, or tumor tissue, from a  
subject afflicted with carcinoma.

3. The method of claim 1 wherein said first contacting step comprises contacting said sample with a divalent cation such that said CBP binds to CEA present in said sample,

5 said CBP comprising an amino acid sequence set forth in the Sequence Listing as SEQ ID NO:2.

4. The method of claim 1 wherein said second contacting step (c) comprises contacting said sample 10 with an immobilized adsorbent.

5. The method of claim 1 wherein said second contacting step (c) comprises contacting said sample with an immunoadsorbent comprising an antibody to CEA.

15 6. The method of claim 5 wherein said second contacting step (c) comprises contacting said sample with an immunoadsorbent comprising a monoclonal antibody to CEA.

20 7. The method of claim 1 wherein said dissociation step comprises contacting said adsorbent-bound conjugate with an agent that chelates said divalent cation such as ethylenediaminetetraacetic acid or 25 ethylene glycol-bis[ $\beta$ -aminoethyl ether]-N,N,N',N'-tetraacetic acid.

-37-

8. The method of claim 1 wherein said collecting step comprises executing a purification method selected from the group consisting of high pressure liquid chromatography, SDS-polyacrylamide gel 5 electrophoresis, affinity chromatography, exclusion chromatography, ammonium sulfate precipitation, ultracentrifugation, isoelectric focusing, and combinations thereof.

10 9. A protein that specifically binds carcinoembryonic antigen (CEA), or a CEA-binding fragment of said protein,

15 said CEA-binding protein (CBP) having a molecular weight of about 20 kD as determined by SDS-polyacrylamide gel electrophoresis, and binding CEA in the presence of a divalent cation in vitro.

10. The protein of claim 1 comprising the amino acid sequence set forth in the Sequence Listing as 20 SEQ ID NO:2.

11. A protein that specifically binds carcinoembryonic antigen (CEA), or a CEA-binding fragment of said protein,

25 said CEA-binding protein (CBP) having a molecular weight of about 21 kD as determined by SDS-polyacrylamide gel electrophoresis, being glycosylated, and binding CEA in the presence of a divalent cation in vitro.

30 12. The protein of claim 11 comprising the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:4.

13. The protein or fragment of claim 11 wherein said 21 kD polypeptide is glycosylated as determined by periodic acid-Schiff base staining.

5 14. The protein or fragment of claim 9 or 11 further comprising a label, such as a radioactive isotope, enzyme, stable free radical, coenzyme, fluorescent group, chemiluminescent group, toxin, or colorimetric group, attached thereto.

10

15. A device comprising:

(a) a support; and

15 (b) at least a CEA-binding fragment of said CBP of claim 9 or 11 bound to said support.

- 39 -

16. A method of detecting carcinoma comprising the  
steps of:

5 (a) administering to a subject a  
pharmaceutical formulation comprising a  
carcinoembryonic antigen binding polypeptide  
(CBP), or a carcinoembryonic antigen binding  
fragment thereof, in a physiologically  
acceptable carrier,

10 said CBP having a molecular weight of  
approximately 20,000 daltons on  
SDS-polyacrylamide gels, and binding CEA in  
the presence of a divalent cation in vitro;

15 (b) obtaining a biological sample such as  
whole blood, serum, bile, saliva, sputum,  
lymphoid tissue, tumor tissue, or ascites  
fluid from said subject;

20 (c) measuring the concentration of CEA in  
said biological sample; and

25 (d) comparing said CEA concentration with a  
predetermined threshold concentration of CEA  
indicative of the presence of carcinoma.

17. The method of claim 16 wherein said  
administering step comprises administering to the  
circulation of said subject a pharmaceutical  
30 formulation including a CBP comprising the amino acid  
sequence set forth in the Sequence Listing as SEQ ID  
NO:2.

-40-

18. A method of detecting carcinoma comprising the  
steps of:

5 (a) administering to a subject a pharmaceutical formulation comprising a carcinoembryonic antigen binding polypeptide (CBP), or a carcinoembryonic antigen binding fragment thereof, in a physiologically acceptable carrier,

10 said CBP having a molecular weight of approximately 21,000 daltons on SDS-polyacrylamide gels, being glycosylated, and binding CEA in the presence of a divalent cation in vitro;

15 (b) obtaining a biological sample such as whole blood, bile, saliva, sputum, lymphoid tissue, tumor tissue, or ascites fluid from said subject;

20 (c) measuring the concentration of CEA in said biological sample; and

25 (d) comparing said CEA concentration with a predetermined threshold concentration of CEA indicative of the presence of carcinoma.

19. The method of claim 18 wherein said administering step comprises administering to the circulation of said subject a pharmaceutical formulation including a CBP comprising the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:4.

-41-

20. The method of claim 16 or 18 wherein said administering step comprises administering said formulation to said subject by injection.

5 21. The method of claim 16 or 18 wherein said measuring step further comprises conducting an assay for carcinoembryonic antigen on said sample.

22. The method of claim 21 wherein said conducting 10 step further comprises conducting an immunoassay for carcinoembryonic antigen.

23. A method of treating carcinoma comprising the steps of:

15

20

(a) providing a carcinoembryonic antigen binding protein (CBP) or fragment thereof which binds carcinoembryonic antigen (CEA) in the presence of a divalent cation, such as  $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$ , or  $\text{Mg}^{+2}$ , in vitro, said CBP having a molecular weight on polyacrylamide gels of about 20,000 daltons; and

25

(b) administering said CBP in a pharmaceutically acceptable carrier to a subject afflicted with carcinoma,

30 said CBP binding CEA, thereby inhibiting the metastatic proliferation of carcinoma cells.

30

24. The method of claim 23 wherein said providing step comprises providing a CBP comprising an amino acid sequence set forth in the sequence listing as SEQ ID NO:2.

35

-42-

25. A method of treating carcinoma comprising the  
steps of:

5 (a) providing a carcinoembryonic antigen  
binding protein (CBP) or fragment thereof  
which binds carcinoembryonic antigen (CEA)  
in the presence of a divalent cation in  
vitro, said CBP having a molecular weight on  
polyacrylamide gels of about 21,000 daltons  
10 and being glycosylated; and

15 (b) administering said CBP in a  
pharmaceutically acceptable carrier to a  
subject afflicted with carcinoma,

said CBP binding CEA, thereby inhibiting the  
metastatic proliferation of carcinoma cells.

26. The method of claim 25 wherein said providing  
20 step comprises providing a CBP comprising an amino  
acid sequence set forth in the sequence listing as  
SEQ ID NO:4.

27. The method of claim 23 or 25 wherein said  
25 providing step comprises purifying said CBP from a  
subject inflicted with carcinoma.

28. The method of claim 23 or 25 wherein said providing step comprises:

- (a) obtaining a biological sample from a subject inflicted with carcinoma, said sample selected from the group consisting of ascites fluid, serum, whole blood, bile, saliva, sputum, lymphoid tissue, and tumor tissue;
- (b) treating said sample with a divalent cation, such as  $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$ , or  $\text{Mg}^{+2}$ , at a concentration and for a time sufficient to enable the binding of said CBP to CEA, thereby forming a CBP-CEA conjugate;
- (c) contacting said sample with an adsorbent that binds CEA for a time sufficient to allow said conjugate to adsorb to said adsorbent;
- (d) removing a portion of said sample which have not adsorbed to said adsorbent;
- (e) dissociating said CBP from said adsorbent-bound CBP-CEA conjugate; and
- (f) collecting said dissociated CBP.

29. An antibody or fragment thereof that recognizes a carcinoembryonic antigen (CEA)-binding protein or CEA-binding fragment thereof,

5 said CEA-binding protein having a molecular weight of about 20 kD as determined by SDS-polyacrylamide gel electrophoresis, and binding CEA in the presence of a divalent cation in vitro.

30. An antibody or fragment thereof that recognizes 10 a carcinoembryonic antigen (CEA)-binding protein or CEA-binding fragment thereof,

15 said CEA-binding protein having a molecular weight of about 21 kD as determined by SDS-polyacrylamide gel electrophoresis, is glycosylated, and binding CEA in the presence of a divalent cation in vitro.

31. The antibody of claim 29 or 30 wherein said antibody is a monoclonal antibody.

-45-

32. A method of detecting carcinoma comprising the  
steps of:

5 (a) subjecting a biological sample from a  
subject to a test indicating the presence of  
a cancer marker,

10 said marker comprising a  
carcinoembryonic antigen binding protein  
(CBP) that binds carcinoembryonic antigen  
(CEA) in the presence of a divalent cation  
in vitro, and has a molecular weight of  
about 20,000 daltons on SDS-polyacrylamide  
gels, and

15 (b) screening said sample for the presence  
of said CBP,

the presence of said CBP being indicative of  
the presence of carcinoma in said subject.

20 33. The method of claim 32 wherein said subjecting  
step further comprises subjecting a biological sample  
to a test indicating the presence of a cancer marker,  
said marker comprising the amino acid sequence set  
25 forth in the Sequence Listing as SEQ ID NO:2.

34. A method of detecting carcinoma comprising the  
steps of:

5 (a) subjecting a biological sample from a  
subject to a test indicating the presence of  
a cancer marker,

10 said marker comprising a  
carcinoembryonic antigen binding protein  
(CBP) that binds carcinoembryonic antigen  
(CEA) in the presence of a divalent cation  
in vitro, and has a molecular weight of  
about 21,000 daltons on SDS-polyacrylamide  
gels, and is glycosylated; and

15 (b) screening said sample for the presence  
of said CBP,

the presence of said CBP being indicative of  
the presence of carcinoma in said subject.

20 35. The method of claim 34 wherein said subjecting  
step further comprises subjecting a biological sample  
to a test indicating the presence of a cancer marker,  
said marker comprising the amino acid sequence set  
25 forth in the Sequence Listing as SEQ ID NO:4.

36. The method of claim 32 or 34 wherein said  
subjecting step further comprises subjecting a  
biological sample, such as whole blood, serum, bile,  
30 saliva, sputum, lymphoid tissue, tumor tissue, or  
ascites fluid, to an immunoassay.

-47-

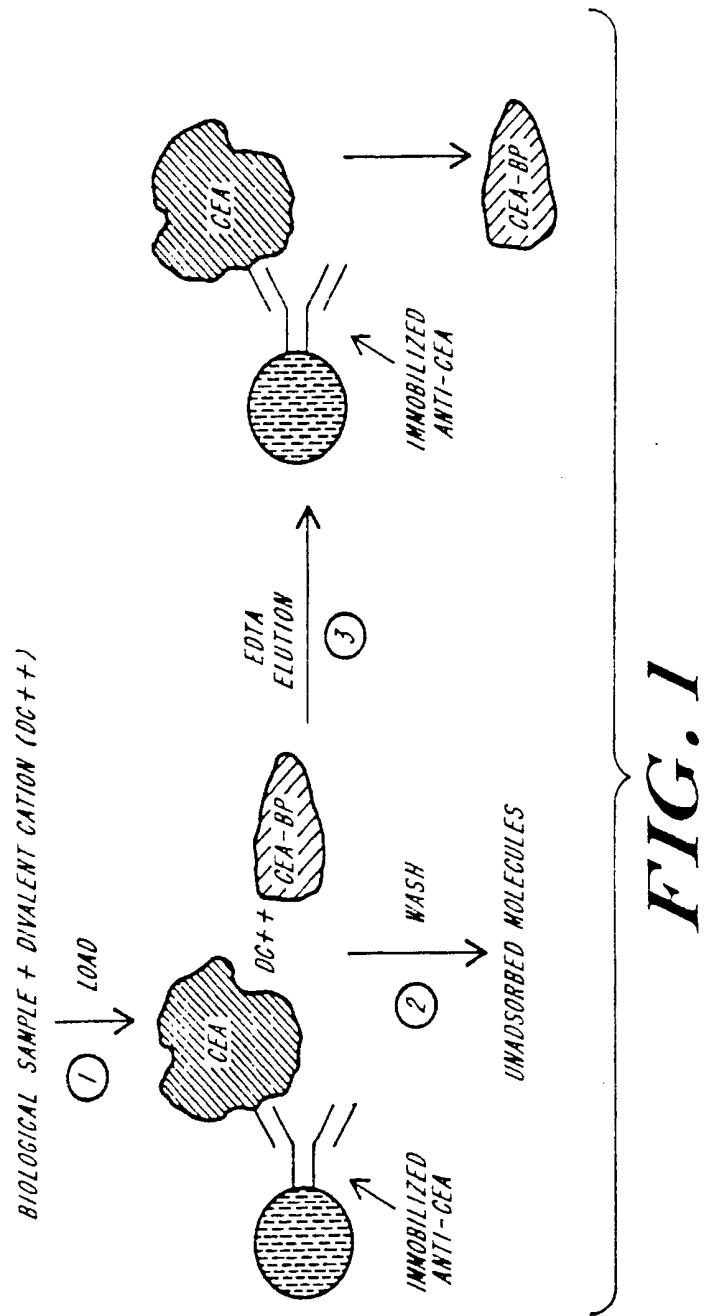
37. A kit for screening a patient for carcinoma, said kit comprising an antibody specific for a carcinoembryonic antigen binding protein (CBP) or a CBP-binding fragment thereof, said CBP having a 5 molecular weight of about 20,000 daltons on SDS-polyacrylamide gels, and binding carcinoembryonic antigen (CEA) in the presence of a divalent cation in vitro,

10 said kit further comprising an antibody or binding fragment thereof specific for a carcinoma marker selected from the group consisting of carcinoma orosomucoid-related antigen (CORA), carcinoembryonic antigen (CEA), CA 19.9, non-specific cross-reacting antigen (NCA), and alpha-1 acid 15 glycoprotein (AGP).

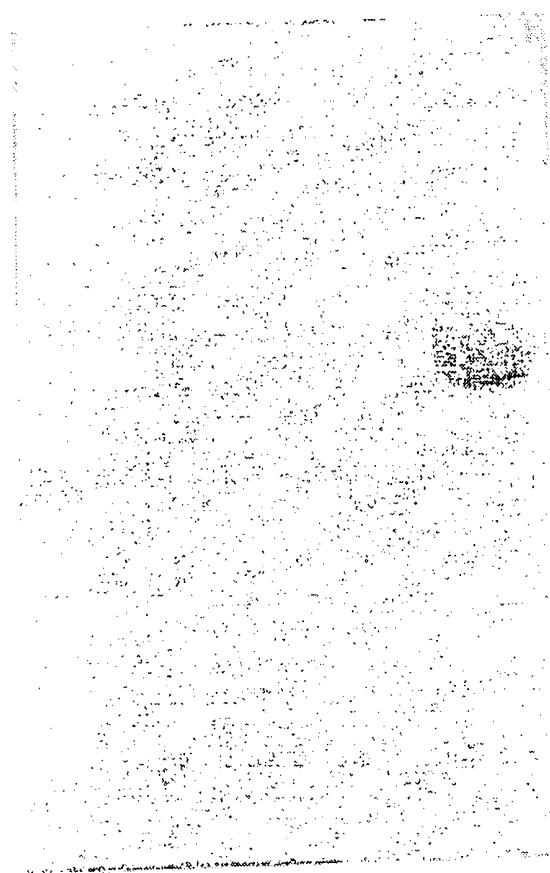
38. A kit for screening a patient for carcinoma, said kit comprising an antibody specific for a carcinoembryonic antigen binding protein (CBP) or a 20 CBP-binding fragment thereof, said CBP having a molecular weight of about 21,000 daltons on SDS-polyacrylamide gels, being glycosylated, and binding carcinoembryonic antigen (CEA) in the presence of a divalent cation in vitro,

25 said kit further comprising an antibody or binding fragment thereof specific for a carcinoma marker selected from the group consisting of carcinoma orosomucoid-related antigen (CORA), carcinoembryonic antigen (CEA), CA 19.9, non-specific 30 cross-reacting antigen (NCA), and alpha-1 acid glycoprotein (AGP).

39. The kit of claim 37 or 38 wherein said antibody specific for said CBP is a monoclonal antibody.

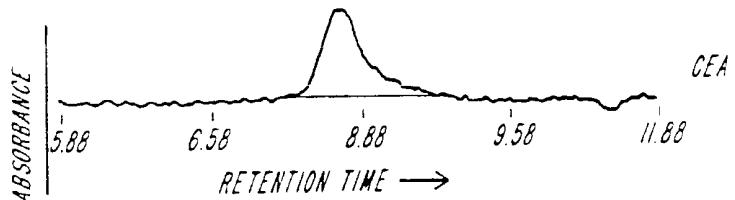


2/8

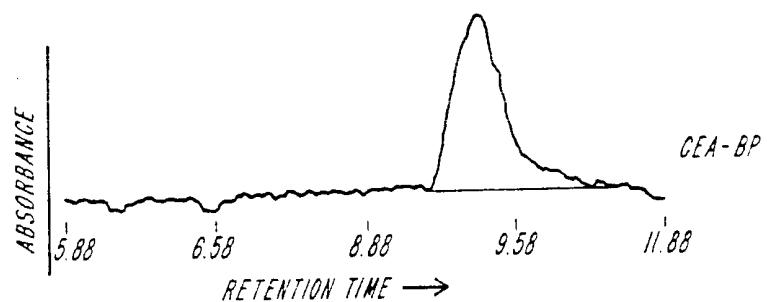


*FIG. 2*

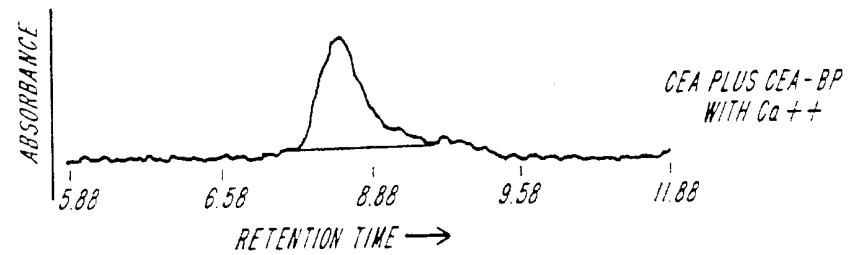
3/8



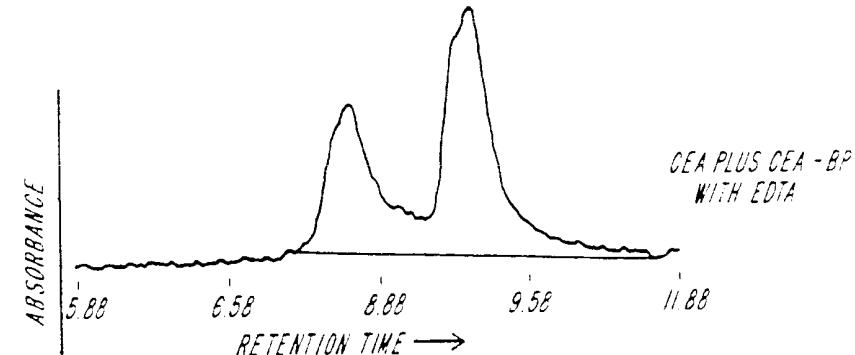
*FIG. 3A*



*FIG. 3B*

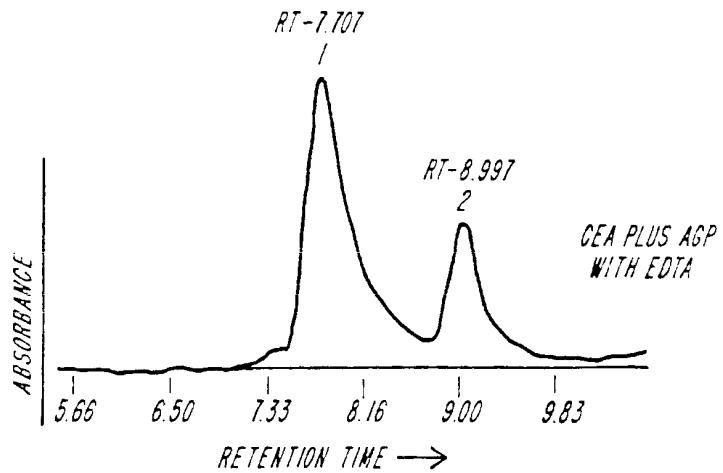


*FIG. 3C*

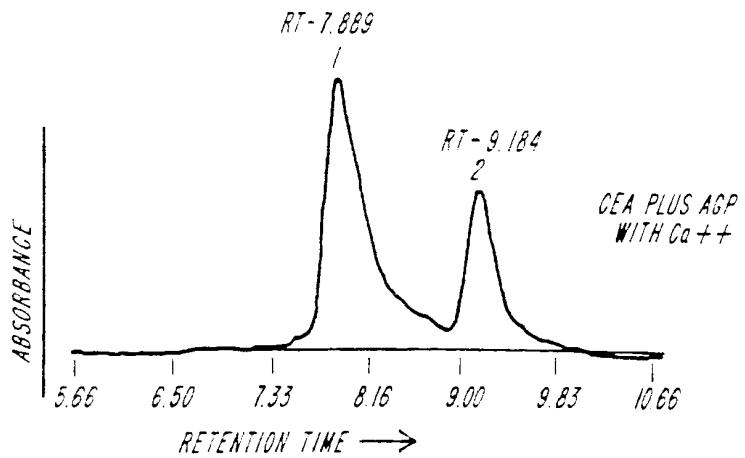


*FIG. 3D*

4/8



**FIG. 4A**



**FIG. 4B**

COMPARISON OF CRP and the 20 KD and 21 KD CBP

Met Glu Lys Leu Leu Leu Cys Phe Leu Val Leu  
 1 5 10

21 KD CBP --> \* Thr Asp  
 Thr Ser Leu Ser His Ala Phe Gly Gln Thr Asp  
 20 KD CBP --> Phe Gly Gln  
 15 20

Leu Ser Gly Lys Val Phe Val Phe Pro \* Glu  
 Met Ser Arg Lys Ala Phe Val Phe Pro Lys Glu  
 Met Gly Gly Lys Ala Phe Val Phe Pro Lys Ser  
 25 30

Leu Ile Thr Pro  
 Ser Asp Thr Ser Tyr Val Ser Leu Lys Ala Pro  
 35 40

Leu Glu Lys Pro Leu Gln \* Phe Thr \* Ser  
 Leu Thr Lys Pro Leu Lys Ala Phe Thr Val Cys  
 45 50 55

\* \* Ala Tyr  
 Ley His Phe Tyr Thr Glu Leu Ser Ser Thr Arg  
 60 65

Gly Tyr Ser Ile Phe Ser Tyr Ala Thr Lys Arg  
 70 75

Gln Asp Asn Glu Ile Leu Ile Phe Trp Ser Lys  
 80 85

Asp Ile Gly Tyr Ser Phe Thr Val Gly Gly Ser  
 90 95

Glu Ile Leu Phe Glu Val Pro Glu Val Thr Val  
 100 105 110

**FIG. 5A**

6/8

Ala Pro Val His Ile Cys Thr Ser Trp Glu Ser  
115 120

Ala Ser Gly Ile Val Glu Phe Trp Val Asp Gly  
125 130

Lys Pro Arg Val Arg Lys Ser Leu Lys Lys Gly  
135 140

Tyr Thr Val Gly Ala Glu Ala Ser Ile Ile Leu  
145 150

Gly Gln Glu Gln Asp Ser Phe Gly Gly Asn Phe  
155 160 165

Glu Gly Ser Gln Ser Leu Val Gly Asp Ile Asp  
170 175

Asn Val Asn Met Trp Asp Phe Val Leu Ser Pro  
180 185

Asp Glu Ile Asn Thr Ile Tyr Leu Gly Gly Pro  
190 195

Phe Ser Pro Asn Val Leu Asn Trp Arg Ala Leu  
200 205

Lys Tyr Glu Val Gln Gly Glu Val Phe Thr Lys  
210 215 220

Pro Gln Leu Trp Pro  
225

NOTE: \* indicates amino acids which the computer  
could not identify, most probably Cys, His, Arg, or  
Trp.

*FIG. 5B*

COMPARISON OF SAP and the 20 kD and 21 kD CBP

Met Asn Lys Pro Leu Leu Trp Ile Ser Val Leu  
1 5 10

21 kD CBP --> \* Thr Asp  
Thr Ser Leu Leu Glu Ala Phe Ala His Thr Asp  
20 kD CBP --> Phe Gly Gln Thr Leu  
15 20

Leu Ser Gly Lys Val Phe Val Phe Pro \* Glu  
Leu Ser Gly Lys Val Phe Val Phe Pro Arg Glu  
Met Gly Lys Ala Phe Val Phe Pro Lys Ser  
25 30

Ser Val Thr Asp \* Val Asn Leu Ile Thr Pro  
Ser Val Thr Asp His Val Asn Leu Ile Thr Pro  
35 40

Leu Glu Lys Pro Leu Gln \* Phe Thr \* Ser  
Leu Glu Lys Pro Leu Gln Asn Phe Thr Leu Cys  
45 50 55

\* \* Ala Tyr  
Phe Arg Ala Tyr Ser Asp Ley Ser Arg Ala Tyr  
60 65

Ser Leu Phe Ser Tyr Asn Thr Gln Gly Arg Gly  
70 75

Asn Glu Leu Leu Val Tyr Lys Glu Arg Val Gly  
80 85

Glu Tyr Ser Leu Tyr Ile Gly Arg His Lys Val  
90 95

Thr Ser Lys Val Ile Glu Lys Phe Pro Ala Pro  
100 105 110

*FIG. 6A*

Val His Ile Cys Val Ser Trp Glu Ser Ser Ser  
115 120

Gly Ile Ala Glu Phe Trp Ile Asn Gly Thr Pro  
125 130

Leu Val Lys Lys Gly Leu Arg Gln Gly Tyr Phe  
135 140

Val Gln Ala Gln Pro Lys Ile Val Leu Gln Gly  
145 150

Glu Gln Asp Ser Tyr Gly Gly Lys Phe Asp Arg  
155 160 165

Ser Gln Ser Phe Val Gly Glu Ile Gly Asp Leu  
170 175

Tyr Met Trp Asp Ser Val Leu Pro Pro Glu Asn  
180 185

Ile Leu Ser Ala Tyr Gln Gly Thr Pro Leu Pro  
190 195

Ala Asn Ile Leu Asp Trp Gln Ala Leu Asn Tyr  
200 205

Glu Ile Arg Gly Tyr Val Ile Ile Lys Pro Leu  
210 215 220

Val Trp Val

NOTE: \* indicates amino acids which the computer  
could not identify, most probably Cys, His, Arg, or  
Trp.

*FIG. 6B*

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/04353

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) - Please See Extra Sheet.

US CL : 436/64, 503; 514/12; 530/350, 388.1, 412

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/64, 503, 543, 544; 514/8, 12, 13, 21; 530/350, 388.1, 395, 412, 413, 415, 828

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, search terms: cca, carcinoembryonic; GENESEQ, PIR 29, SWISS-PROT, search terms: SEQ ID NOS 2 and 4.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal Of Biological Chemistry, Vol. 260, No. 24, issued 25 October 1985, Prelli et al., "The Primary Structure Of Human Tissue Amyloid P Component From A Patient With Primary Idiopathic Amyloidosis", pages 12895-12898, especially the Abstract.	9,11, 13-15, 29-32, 34, 36-39
Y	European Journal Of Rheumatology And Inflammation, Vol. 5, issued 1982, Pepys, "C-Reactive Protein: A Review Of Its Structure And Function", pages 386-397, especially page 386.	9,11, 13-15, 29-31, 37-39
Y	The Journal Of Biological Chemistry, Vol. 260, No. 22, issued 05 October 1985, Potempa et al., "Effect Of Divalent Metal Ions And pH Upon The Binding Reactivity Of Human Serum Amyloid P Component, A C-Reactive Protein Homologue, For Zymosan", pages 12142-12147, especially page 12143, column 1.	14
Y	US, A, 4,782,014 (Serban et al.) 01 November 1988, column 1, lines 45-54, and column 3, lines 35-41.	29-31,37-39

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*'A'	document defining the general state of the art which is not considered to be of particular relevance	
*'E'	earlier document published on or after the international filing date	"X"
*'L'	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"
*'O'	document referring to an oral disclosure, use, exhibition or other means	"&"
*'P'	document published prior to the international filing date but later than the priority date claimed	document member of the same patent family

Date of the actual completion of the international search

13 August 1992

Date of mailing of the international search report

19 AUG 1992

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/04353

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Scandinavian Journal Of Immunology, Vol. 24, issued 1986, Levo et al., "Serum Amyloid P-Component Levels In Patients With Malignancy", pages 147-151, especially the Abstract.	32,34,36
Y	US, A, 4,892,933 (Salem et al.) 09 January 1990, column 4, lines 45-53.	37-39

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US92/04353

## A. CLASSIFICATION OF SUBJECT MATTER

IPC (5):

A61K 37/02; C07K 3/02, 3/12, 15/06, 15/28; G01N 33/53, 33/58, 33/68